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Mutation of the *S* and *3c* genes in genomes of feline coronaviruses

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Running Head: MUTATION OF FELINE CORONAVIRUS GENOME

ABSTRACT

Feline coronavirus (FCoV) is classified into two biotypes based on its pathogenicity in cats: a feline enteric coronavirus of low pathogenicity and a highly virulent feline infectious peritonitis virus. It has been suspected that FCoV alters its biotype via mutations in the viral genome. The *S* and *3c* genes of FCoV have been considered the candidates for viral pathogenicity conversion. In the present study, FCoVs were analyzed for the frequency and location of mutations in the *S* and *3c* genes from faecal samples of cats in an animal shelter and the faeces, effusions, and tissues of cats that were referred to veterinary hospitals. Our results indicated that approximately 95% FCoVs in faeces did not carry mutations in the two genes. However, 80% FCoVs in effusion samples exhibited mutations in the *S* and *3c* genes with remainder displaying a mutation in the *S* or *3c* gene. It was also suggested that mutational analysis of the *3c* gene could be useful for studying the horizontal transmission of FCoVs in multi-cat environments.

KEYWORDS: feline coronavirus, multi-cat environment, mutation, *S* gene, *3c* gene,

INTRODUCTION

The genome of feline coronavirus (FCoV), a member of the *Alphacoronavirus 1* species of the genus *Alphacoronavirus*, comprises single-stranded positive-sense RNA [9]. FCoV infection is prevalent in cats worldwide and is divided into two biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). The former has low pathogenicity, causing mild enteritis or unapparent infection, and the latter is highly virulent and lethal. FIP is characterised by the accumulation of body cavity effusions (effusive or wet form) and the formation of granulomatous lesions affecting multiple organs (non-effusive or dry form) [10]. FIPVs are considered mutants of FECVs [11,12,14].

Although the viral genes responsible for biotype conversion have not been completely elucidated, the candidate genes have been identified. The *S* gene encodes spike protein on the viral membrane. It was reported that 95.8% of 118 serotype I FIPVs displayed missense mutations in codon 1,058 or 1,060 of the *S* gene, whereas none of the sample of 183 FECVs exhibited these mutations [6]. The non-synonymous mutations in codons 1,058 and 1,060 substituted methionine to leucine (M1,058L) and serine to alanine (S1,060A), respectively. The *3c* gene encoding an accessory viral protein was also reported to be mutated in 60%–100% of FIPVs, resulting in the loss or truncation of the 3c protein, whereas most FECVs carried an intact *3c* gene [3-5,8,11,13,14]. Accordingly, it was considered that mutation of the *S* gene, *3c* gene or both was involved in the acquisition or augmentation of lethal pathogenicity in the majorityof FIPV field strains. In the present study, we analyzed the *S* and *3c* genes of FCoVs detected in faecal materials, effusion samples, and tissues that were obtained from cats in Japan to determine the frequency and location of the mutations. An analysis

of the *3c* gene suggested the horizontal infection of FCoVs, which were detected in effusions and tissues, among several housemate cats in a multi-cat environment.

MATERIALS AND METHODS

Collection of clinical samples

Clinical specimens were obtained from 93 cats referred to private veterinary hospitals in Japan for suspected FIP based on clinical symptoms, including pyrexia, vomiting, diarrhoea, jaundice, emaciation, anaemia, ascites, pleural effusion, ophthalmologic abnormalities, neurological signs, and death. Some animals displayed an enlargement of abdominal organs that was noticed on palpation, radiography, or ultrasound. The samples of abdominal and pleural effusions, whole blood, serum, rectal swabs, faeces, and tissues were sent to our laboratory under refrigeration. Tissues were obtained via autopsy of four cats that had been kept by the same owner and referred to a veterinary hospital. The analyzed tissues included kidneys, mesenteric lymph nodes, a spleen, and an eye and its vitreous humor. Whole blood samples were treated with ethylenediaminetetraacetic acid as an anticoagulant.

Faecal samples were collected from an animal shelter wherein each cat was housed alone or with a few other cats per cage. To prevent the redundant analysis of a cat when ≥2 cats were kept in a single cage, only one faecal sample was taken.

Nucleic acid extraction and complementary DNA synthesis

Total RNA samples were extracted from effusions, supernatants of phosphate-buffered saline-homogenised faecal and rectal swab samples, serum, plasma, and a vitreous humor sample from an eye using a QIAamp® Viral RNA Mini Kit (QIAGEN,

Hilden, Germany) or ISOGEN-LS reagent (NIPPON GENE, Tokyo, Japan). RNA samples of whole blood were extracted using ISOGEN-LS reagent. In some cases, erythrocytes were lysed using 0.2% sodium chloride to isolate leukocytes, and their RNA was extracted using an RNeasy® Mini Kit (QIAGEN) in combination with a QIA shredder (QIAGEN). Tissues were homogenised in ISOGEN reagent (NIPPON GENE) using a TissueRuptor with TissueRuptor disposable probes (QIAGEN). cDNAs were synthesised using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). All reagents and kits were used according to the manufacturers' instructions.

Amplification of the S and 3c genes by reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to amplify the *S* and *3c* genes using GoTaq® Green Master Mix (Promega, Madison, WI, U.S.A.), previously reported primers [1,5,6] and our designed primers (Supplementary Table 1). The primers were used at a final concentration of 0.5 μM. The *S* gene fragments were amplified to determine the FCoV serotype (I or II) in each animal. Amplification of the *3c* and *S* genes, including codons 1,058 and 1,060, via first-round PCR was performed as follows: initial denaturation at 94°C for 2 min; 50 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec; and final extension at 72°C for 7 min. In some cases, the *3c* and *S* genes were amplified via nested RT-PCR, in which a second-round reaction was performed using the same PCR cycle parameters. The *S* gene-based serotyping was carried out together with the *3c* gene amplification under the same reaction protocol or separately via single or nested RT-PCR, wherein the reaction protocol was the same except for a shortened extension time of 20 sec. The PCR products were electrophoresed on a 2% agarose gel and amplified DNA fragments were retrieved

using the Wizard® SV Gel and PCR Clean-Up System (Promega). The extracted product was directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit on a genetic analyzer (Applied Biosystems 3130, Thermo Fisher Scientific, Waltham, MA, U.S.A.). Some amplicons were cloned into a pCR2.1-TOPO vector using a TOPO® TA Cloning® Kit (Thermo Fisher Scientific) and sequenced using M13 primers or the primers used for RT-PCR. The obtained *3c* gene sequences were analyzed to determine the types and locations of mutations via comparisons with type I FECV strains RM (FJ938051) and UU19 (HQ392470) and type II FIPV strain KUK-H/L (AB781789), none of which carry mutations resulting in the production of truncated proteins. GENETYX 13 (Genetyx Corporation, Tokyo, Japan) and BioEdit 7.1.3.0 [7] software were used for sequence analysis. All determined *3c* gene sequences were submitted to the DNA Data Bank of Japan. Accession numbers are shown in Supplementary Tables 2 and 3.

RESULTS

Detection of FCoVs from clinical samples

Of the 53 samples obtained from 40 out of 93 cats that had been referred to animal hospitals, 55 *3c* gene sequences were obtained (Supplementary Table 2). FCoV serotypes I and II were detected in 38 and 2 cats, respectively. The ages of 39 animals with FCoV positivity in any sample ranged from 2 months–17 years (median, 9.5 months), and 30 animals were younger than two years old. Cat 19 was of unknown age. Twenty cats were male, 18 were female and the sex was not recorded for two animals. In stools collected from the animal shelter, *3c* genes were detected in 19 samples (Supplementary Table 3).

133

134 *Analysis of the S gene*

135 Partial *S* gene fragments of FCoV including codons 1,058 and 1,060 were amplified
136 from the faeces of 19 cats from the animal shelter. Codon mutations were not present in
137 all samples. The FCoV *S* gene in faecal samples from 14 cats that had been referred to
138 animal hospitals was also examined. Four of these cats were fed by a single owner, and
139 the M1,058L or S1,060A mutation was detected in their faeces. Half of the 14 hospital
140 cases presented with ascites or pleural effusion in which FCoV genomes were detected.
141 Of the 30 ascites and pleural effusion samples that contained type I FCoVs, M1,058L
142 and S1,060A mutations were discovered in 24 and 4 samples, respectively, and the
143 remaining 2 samples did not carry the mutations.

144 Six tissue samples were obtained from four deceased cats that were 4–6 months of
145 age. All tissues contained FCoVs that carried the M1,058L mutation.

146 An FCoV that was detected in the blood sample from cat 55 had the M1,058L
147 mutation, but because other samples were not taken, this cat was not analyzed further.

148

149 *Analysis of the 3c gene*

150 Previously reported information regarding the open reading frame (ORF) lengths of
151 the *3c* gene was obtained from the National Center for Biotechnology Information
152 online database. The majority of non-truncated ORFs consisted of 714 nucleotides
153 coding 237 amino acids (aa). Some ORFs were longer because of one or more insertions
154 of several nucleotides. Accordingly, in the present study, an intact *3c* ORF was defined
155 as a sequence of at least 714 bases that did not contain a premature stop codon due to
156 any mutation type.

157 The *3c* ORF was 714 bases long in 18 out of 19 FCoV-positive stool samples from
158 the animal shelter. The ORF of the virus detected in the stool sample from cat S10
159 [shelter cat] was 711 bases due to a 3-base deletion spanning codons 23–24, resulting in
160 the deletion of 1 aa. This mutation did not generate a premature stop codon (Fig. 1A).
161 The *3c* genes were also analyzed from the 14 FCoV-containing faecal samples from cats
162 that had been referred to animal hospitals. One faecal FCoV from cat 37 had a longer
163 intact *3c* gene of 720 bases. This sequence was genetically closest (96.3%) in a BLAST
164 search to two intact *3c* gene sequences of FIPV strains DSKUU48 (GU053649) [5] and
165 UU9 [6].
166 Ascites and pleural effusion samples containing FCoVs were taken from 32 cats in
167 animal hospitals. Two ascites samples contained type II FCoVs with truncating
168 mutations in the ORF of *3c*. The other 30 samples were type I FCoVs. Of these, 26
169 samples carried truncating mutations in the *3c* genes (Fig. 1B). Some FCoVs were not
170 expected to express the 3c protein because of a mutation involving the start codon.
171 All FCoVs identified in the six tissue samples of four cats contained a truncated ORF
172 in each *3c* gene (Fig. 1C). In a kidney and mesenteric lymph node from cat 80, two
173 FCoV variants were detected in each tissue, in which the *3c* ORFs were 712 and 684
174 bases, respectively. Both variants shared an identical two-base deletion at codon 153,
175 and one variant had an additional 28-base deletion located 46 bases downstream of the
176 two-base deletion site. An FCoV in blood of cat 55 had an intact *3c* gene.
177 The lengths of truncated 3c proteins expressed by FCoVs were predicted to range
178 from 3 to 235 aa, corresponding to 1.3%–99.2% of the length of the wild-type protein.
179
180 *Mutation types leading to truncation or deletion of the 3c protein*

Mutations that resulted in the production of truncated 3c proteins less than 237 aa or complete protein loss were detected in 39 samples collected from 33 cats. This included one faecal sample from a cat housed in an animal shelter and clinical samples from 32 hospital-referred cats. Two deletions (faeces from cat S10 and ascites from cat 75) and one insertion (faeces from cat 37) did not create premature stop codons. The other *3c* genes amplified from 37 samples of 31 cats had mutations resulting in premature stop codons or no protein expression because of a mutation that involved the start codon of each sequence. The most common mutation type that generated premature stop codons was a frameshift resulting from a deletion or insertion (18 samples [48.6%] from 16 cats). Deletions accounted for the majority (17 of 18 samples) of the frameshifts. The second most common cause of premature termination was a nonsense mutation (15 samples [40.5%] from 11 cats). A missense mutation at the start codon was found in three samples (8.1%) from three cats, and an ATG codon next to the original start codon in each sequence was out of frame in all three samples. Deletion of a region including the start codon was found in one sample (2.7%).

Relationship of the mutation of S and 3c genes

The relationship of *S* and *3c* gene mutations in each sample type is indicated in Table 1. For FCoVs in 19 faecal samples that were obtained from the animal shelter, no viruses carried missense mutations at codons 1,058 and 1,060 of the *S* gene. Only one sample showed a deletion of three consecutive nucleotides in the *3c* gene, causing the lack of one aa.

In the four cats belonging to a single owner, the faecal samples contained FCoVs where the M1,058L mutation was found together with truncating mutations of the *3c*

genes. FCoVs in the other ten hospital samples did not carry mutations in the *S* and 3c genes.

In the effusion samples, type I FCoVs had mutations in both the *S* and *3c* genes in 24 of 30 samples. A mutation at either codon 1,058 or 1,060 was present in 4 out of 30 samples. The remaining two effusion samples carried only *3c* gene truncating mutations. The present study detected two type II FCoVs in ascites samples, both viruses carrying truncating mutations in the *3c* gene. In the tissue samples of the four cats that belonged to one owner, all FCoVs in tissues had both the M1,058L mutation and *3c* gene truncating mutation.

Sequence relationship among co-habitants

Six cats included in this study were co-habitants (80, 81, 82, 85, 87 and 88) that were fed by a single owner. Cats 80–82 were 4-month-old littermates that died within a month of disease onset and were autopsied. Cat 87 died approximately 2 months later and was also autopsied. Consequently, 14 samples including faecal samples and rectal swabs from the six cats were analyzed, and some identical and closely related mutations were identified (Fig. 2). The ORF homology among the samples ranged from 95.66%–99.86%.

A 714-base consensus sequence generated from these samples was identical to the *3c* gene of an FCoV in a rectal swab from cat 80. Deletion of the second and third nucleotides at codon 153 was found in FCoVs detected in the lymph node and kidney tissues of cat 80. An identical deletion was shared in a virus detected in ascites from cat 85. A frameshift caused by this deletion resulted in the generation of a premature stop codon. The kidney and lymph node samples of cat 80 demonstrated another virus

variant featuring a 28-base deletion located 45 bases downstream of the two-nucleotide deletion site. An FCoV in a rectal swab from cat 85 had a closely related 29-base deletion at the same position in the *3c* ORF.

A nonsense mutation at codon 210 was identified in the rectal swab and vitreous humor sample from cat 82. The same mutation was detected in viruses in a rectal swab, ascites, and kidney samples from cat 87. The homology of the sequences between the rectal swab from cat 80 and samples from cats 82 and 87 ranged from 99.44%–99.86%. Another nonsense mutation at codon 205 was found in the spleen and lymph node samples of cat 81. A two-nucleotide deletion at codon 123 was detected in an FCoV isolated from the faeces of cat 88.

Cats 7-1 and 7-2 were 3-month-old kittens that were housed together. Both cats displayed the accumulation of ascites and pleural effusion over the same period, and the effusion samples were obtained from the hospital on the same day. The sequence homology of the samples was 99.44%, but premature stop codons were caused by a deletion and frameshift in cat 7-1 and a nonsense mutation in cat 7-2.

DISCUSSION

A previous investigation had determined that 96.2% FIPVs causing wet form FIP had either an M1,058L (89.9%) or S1,060A (6.3%) mutation in the *S* gene [6]. Our present study revealed that type I FCoVs in ascites and pleural effusion samples had the M1,058L and S1,060A missense mutations at a rate of 80.0% and 13.3%, respectively. Because histopathological examinations of the cats were not performed, the biotypes of FCoVs analyzed in this study could not be determined. Therefore, the relationship between the biotypes and gene mutations was not analyzed. However, it is considered

253 that approximately ≥90% FCoVs in effusion samples have one of the *S* gene mutations.
254 On the contrary, neither M1,058L nor S1,060A mutations were found in FCoVs in any
255 of the 19 faecal samples from shelter cats, some of which had soft stools, indicating
256 enteritis. A previous report indicated that none of the FECVs in rectal swabs carried a
257 mutation at codon 1,058 or 1,060 [6]. Accordingly, it is suggested that majority FCoVs
258 in faeces of clinically healthy cats and cats with only mild enteritis carry the *S* genes
259 without any of these mutations. The M1,058L mutation was also detected in faeces and
260 tissues of four young diseased cats that had died. The biotype of the viruses was
261 unknown, but faecal FCoVs with the M1,058L or S1,060A mutation would require
262 experimental infection for pathogenicity determination, even when FIP was confirmed
263 via histopathological examination.

264 Previous studies have identified truncating mutations of the *3c* gene in the genomes
265 of >60% of FIPVs, whereas most FECVs carried intact *3c* genes. Although the precise
266 molecular function of the 3c protein is unknown, it has been reported to play an
267 essential role in FECV replication in the intestines [2,13]. Our present study determined
268 that 87.5% FCoVs in body cavity effusions, which included type I and II viruses, and all
269 type I FCoVs, in six tissues collected from four cats, carried *3c* gene mutations resulting
270 in the truncation or loss of 3c protein. However, such mutations were not detected in the
271 majority of faecal FCoVs of shelter cats that did not exhibit any clinical symptoms
272 except for soft stools. This finding is similar to previously published data [3,6].
273 Therefore, it is considered that in addition to the M1,058L and S1,060A mutations in
274 the *S* gene, a truncating mutation of the *3c* gene is another genetic feature that is
275 relatively characteristic of FCoVs in effusions and tissues. Molecular functional

276 analysis of the 3c protein is required to elucidate the influence of the *3c* gene mutation
277 on the FCoVs.

278 The M1,058L or S1,060A mutations were found along with a *3c* gene truncating
279 mutation in 80.0% type I FCoVs from 30 effusion samples, whereas the mutation of
280 either the *S* or *3c* gene was detected in 13.3% and 6.7% of the FCoVs in effusions,
281 respectively. Because the *S* genes were not mutated and *3c* genes were intact in the
282 majority of faecal FCoVs, it is suggested that a mutation of either or both the genes are
283 involved in the alteration of tissue tropism of FCoVs.

284 A frameshift due to a deletion and a nonsense mutation is the most common
285 truncating mutation of the *3c* gene. This result correlated with previous studies [3,5].
286 Interestingly, 22.5% *3c* gene ORFs contained two or three deletions at different
287 positions in each sequence. For example, the *3c* sequence of an FCoV in ascites from
288 cat 35 had a 25-base deletion and another downstream two-base deletion. Therefore, it
289 is considered that mutations often accumulate in the *3c* gene of an FCoV in effusions.

290 FIPV is not usually considered to transmit horizontally [10]. However, in an outbreak
291 of FIP in an animal shelter in Taiwan, an identical nonsense mutation at codon 210 in
292 the 714-base *3c* ORF was shared by serotype II FCoVs in the effusions of two cats that
293 died from FIP within a 5-month interval [15]. Our present research analyzed six
294 housemate cats that were referred to a veterinary hospital. All cats died within 3 months
295 with or without ascites and granulomatous lesions in organs upon gross examination.
296 Some identical mutations were shared in the *3c* gene sequences with >99% homology.
297 Accordingly, it is considered that an analysis of the *3c* genes is useful to determine
298 whether a specific viral strain horizontally transmits among cats.

In the present study, it was shown that approximately 95% faecal FCoVs in an animal shelter had an intact *3c* gene and that the *S* gene that was not mutated at codons 1,058 and 1,060. In contrast, all FCoVs in effusion samples carried a mutation in either or both of the *S* and *3c* genes. Determination of FCoV biotypes is required to elucidate the correlation of pathogenicity of the virus to gene mutations. Further investigations also need the analysis of protein function alterations caused by the mutations.

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FIGURE LEGENDS

Fig. 1. Schematic representation of feline coronavirus *3c* gene sequences determined in this study. 'Wild-type' indicates a sequence encoding 237 amino acids (aa), which is present in most feline enteric coronaviruses. Every sequence shown below contains a mutation resulting in loss, truncation or elongation of the 3c protein in comparison with the wild-type sequence. Arabic numerals on the left indicate the number assigned to the cat. S10 was a cat from an animal shelter. The virus serotype in cat 1 and cat 58 was II, as indicated in parentheses. Each sequence is shown as a horizontal line with the number of nucleotides (nt) and predicted number of amino acids (aa). A deletion is indicated by a white break with 'Δ' and the number of deleted nt. An insertion (Ins) is shown at the insertion position with the number of inserted nucleotides. An asterisk (*) denotes a stop codon. Black colour indicates the portion of the sequence expected to be translated. Grey colour denotes the portion of the sequence that will not be translated due to a premature stop codon. The sequences are arranged in order of descending protein length for each sample type. An effusion sample from cat 33 contained two variant viruses, one of which harbored an nt substitution in the start codon. Abbreviations: Kid., kidney; Vit., vitreous humor; Spl., spleen; L.N., lymph node.

Fig. 2. Sequence similarity of *3c* genes in viruses of cats living in a multi-cat environment. A consensus sequence (top) was determined from all *3c* gene ORFs via software analysis. Each short vertical line on the sequence diagram represents the replacement of a nucleotide from the consensus sequence. Deletions and nonsense mutations, which are identical in length and position, are enclosed by solid lines. A 29-base deletion, closely related to the 28-base deletions indicated, is enclosed by a dashed-

384 line box. Abbreviations: R.S., rectal swab; Kid., kidney; Asc., ascites; Vit., vitreous
385 humor; Sp., spleen, L.N., lymph node; fec., faeces.
386

Fig. 1
Oguma *et al.*

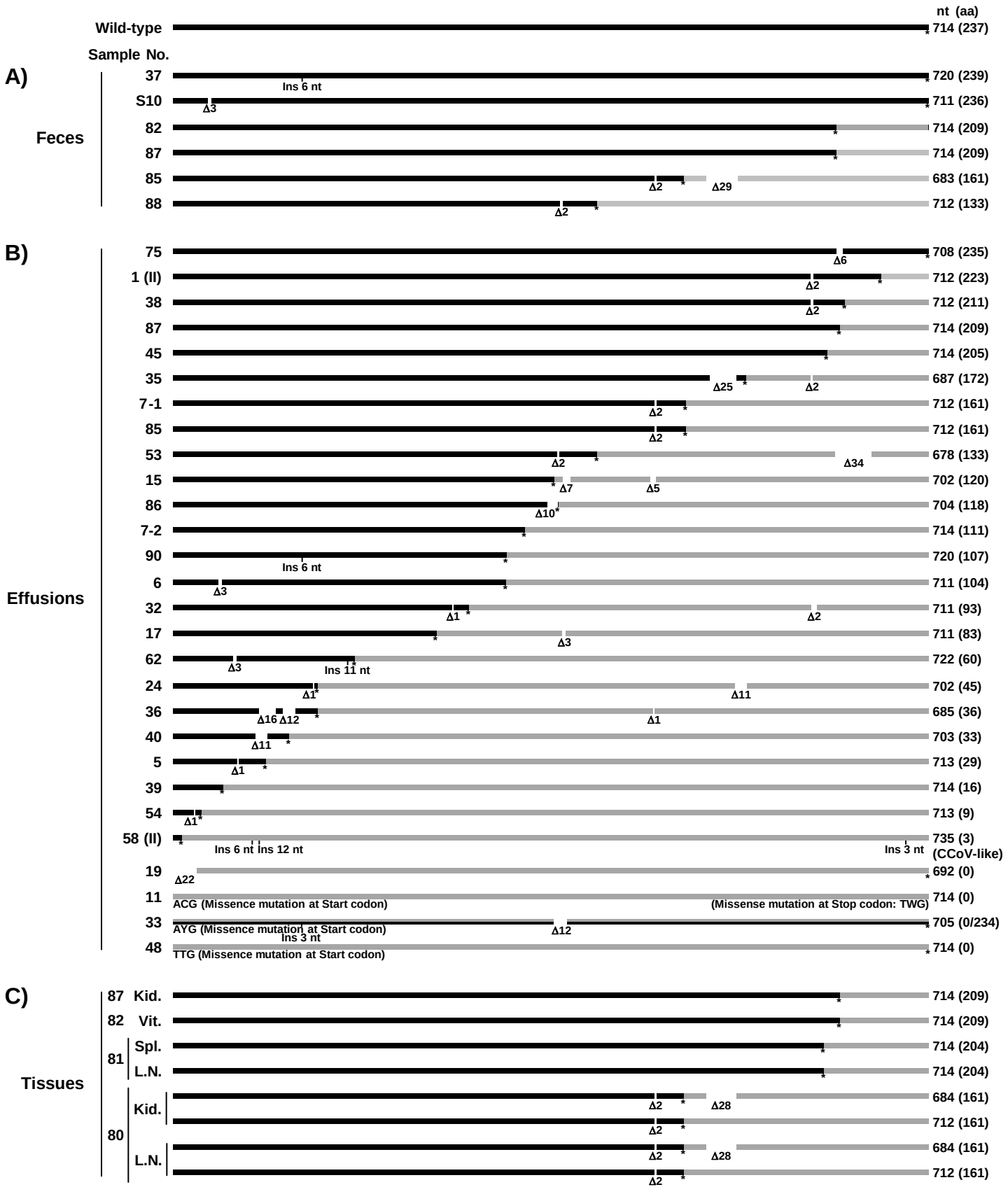

Fig. 2
Oguma *et al.*

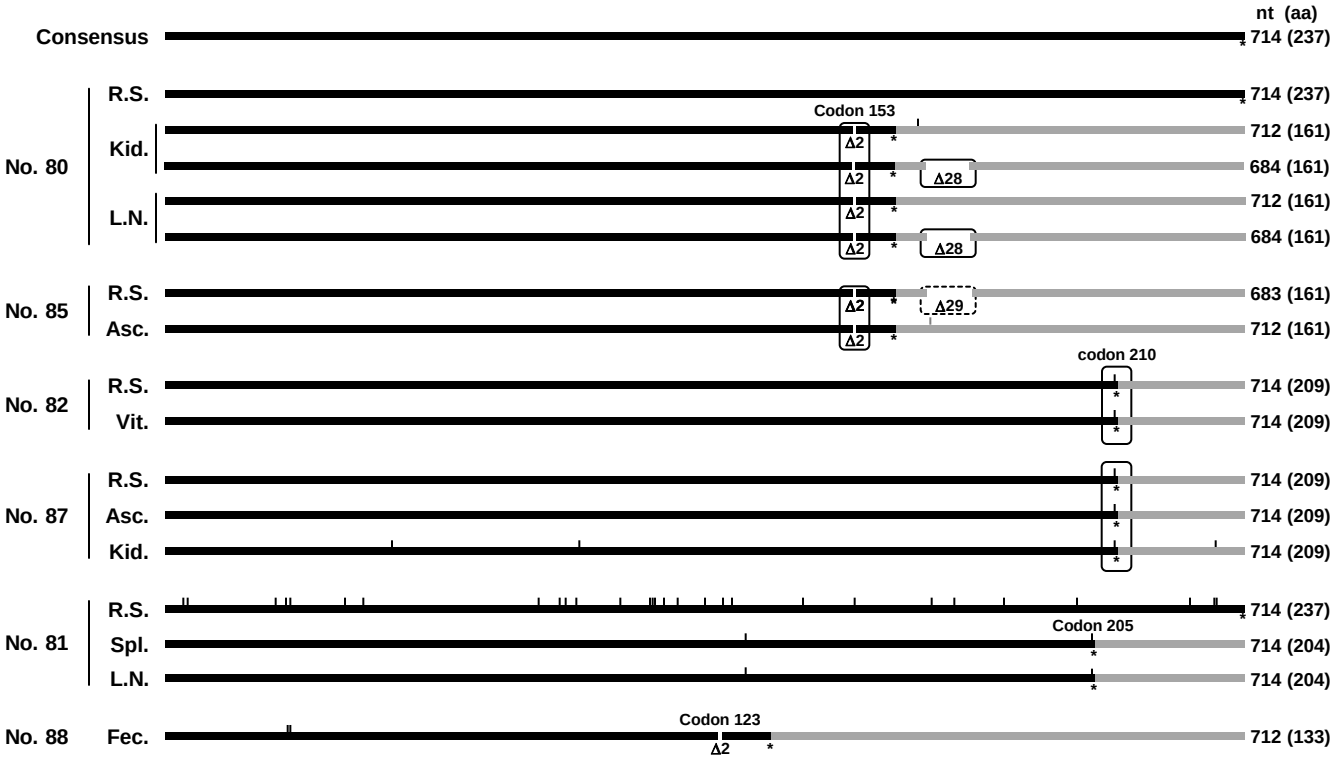

Table 1. Relation of mutatons between S and 3c gene.

Mutated gene in type I FCoVs	No mutation	S gene[a)]	3c gene[b)]	S and 3c genes	(Total)
Faeces of shelter cats	18	0	1	0	19
(n=19)	94.7%	0.0%	5.3%	0.0%	100.0%
Faeces of hospital cats	10	0	0	4	14
(n=14)	71.4%	0.0%	0.0%	28.6%	100.0%
Effusions of hospital cats	0	4	2	24	30
(n=30)	0.0%	13.3%	6.7%	80.0%	100.0%
Tissue of hospital cats	0	0	0	6	6
(n=6, 4 cats)	0.0%	0.0%	0.0%	100.0%	100.0%
Blood of a hospital cat	0	1	0	0	1
(n=1)	0.0%	100.0%	0.0%	0.0%	100.0%
Mutated gene in type II FCoVs	No mutation		3c gene		(Total)
Effusions of hospital cats	0	-	2	-	2
(n=2)	0.0%	-	100.0%	-	100.0%

a) Mutation at codon 1,058 or 1,060

b) Truncating mutation

Supplementary Table 1. Primers used for RT-PCR of FCoV 3c and S gene.

Amplified gene	Primer pair	Orientation	Sequence (5' to 3')	Nt position in FCoVs	Amplicon size (base pair)	Reference
3c	3c-F1	Forward	CAAGTACTATAAAACGTAGAAGMAG	25,061-25,085[a]	845[a]	Chang *et al.*, 2010
	3c-R1	Reverse	CAGGAGCCAGAAGAAGACACTAA	25,883-25,905[a]		Chang *et al.*, 2010
	3c-F2	Forward	GTGTGTATAGGTTTTGGTGA	24,867-24,886[a]	1,080[a]	*[e]
	3c-R2	Reverse	TTTAGCAATGCTATTGAAAA	25,927-25,946[a]		*[e]
	3c-F3	Forward	YTGGTAYAARYTACCTTTTG	24,999-25,018[a]	948[a]	*[e]
	3c-R2		Described above			*[e]
	3c-F4[c]	Forward	GTAGTAGAAGACAATTTGAA	24,509-24,528[b]	1,476[b]	*[e]
	3c-R3[c]	Reverse	TCATTTTGTTTAGTTCAAAC	25,965-25,984[b]		*[e]
	3c-F5[c]	Forward	GGAAGTTGTTGTCACTCTAT	24,487-24,506[b]	1,322[b], [c]	*[e]
	3c-R4[c]	Reverse	CAATATAATTATCAACAGGA	25,789-25,808[b]		*[e]
	3c-F4[c]		Described above		1,300[b], [c]	*[e]
	3c-R4[c]		Described above			*[e]
S	Iffs	Forward	GTTTCAACCTAGAAAGCCTCAGAT	24,201-24,224[a]	376[a]	Addie *et al.*, 2003
(Serotyping)	Icfs	Forward	GCCTAGTATTATACCTGACTA	24,114-24,134[b]	283[b]	Addie *et al.*, 2003
	Iubs	Reverse	CCACACATACCAAGGCC	24,560-24,576[a]		Addie *et al.*, 2003
				24,380-24,396[b]		
	nIffles	Forward	CCTAGAAAGCCTCAGATGAGTG	24208-24229[a]	360[a]	Addie *et al.*, 2003
	nIcfs	Forward	CAGACCAAACTGGACTGTAC	24,177-24,196[b]	211[b]	Addie *et al.*, 2003
	nIubs	Reverse	CCAAGGCCATTTTACATA	24,550-24,567[a]		Addie *et al.*, 2003
				24,370-24,387[b]		
	S-I-F1	Forward	TGACGGCATGGTCAGGAATA	24,089-24,108[a]	475[a]	*[e]
	S-II-F1	Forward	AACTATGTATCAGCCTAGAG	24,021-24,040[b]	363[b]	*[e]
	S-R1	Reverse	GGCCATTTYACATAAGTTTC	24,544-24,563[a]		*[e]
				24,364-24,383[b]		
	S-I-F2	Forward	TATGCATATGTGTTGAAAGA	24,124-24,143[a]	425[a]	*[e]
	S-II-F2	Forward	AGTTCTGATTTTGTTCAAAT	24,049-24,068[b]	320[b]	*[e]
	S-R2	Reverse	GTTTCAATYCTRTTGAGCCA	24,529-24,548[a]		*[e]
				24,349-24,368[b]		
S	Chang-EID2012-F1[d]	Forward	CAATATTACAATGGCATAATGG	23,392-23,413	615[a]	Chang *et al.*, 2012
(Codon 1058 & 1060)	Chang-EID2012-R1[d]	Reverse	CCCTCGAGTCCCGCAGAAACCATACCTA	23,979-24,006		Chang *et al.*, 2012
	Chang-EID2012-F2[d]	Forward	GGCATAATGGTTTTACCTGGTG	23,404-23,425	143[a]	Chang *et al.*, 2012
	Chang-EID2012-R2[d]	Reverse	TAATTAAGCCTCGCCTGCACTT	23,525-23,546		Chang *et al.*, 2012
	Codon1058-F1	Forward	CYTCARCTTGTCARACAATHGAAAAT	23,069-23,094	1,046[a]	*[e]
	Codon1058-R1	Reverse	AACACATATTCCTGACCATG	24,095-24,114		*[e]
	Codon1058-F2	Forward	GCTTGTCADACAATYGAAAATKCCCT	23,074-23,099	982[a]	*[e]
	Codon1058-R2	Reverse	TGAAAGAAAAGYAAACCATCAGGTGC	24,031-24,056		*[e]
	Codon1058-F3	Forward	GATGAYGAYTATAARAAGTG	23,335-23,354	274[a]	*[e]
	Codon1058-R3	Reverse	CARACAATHGAAAATGCCCT	23,589-23,608		*[e]
	Codon1058-F4	Forward	TRTTGAARGCATTAGCAAGT	23,080-23,099	704[a]	*[e]
	Codon1058-R4	Reverse	ATAGCCTGRAARTTTTCTG	23,764-23,783		*[e]

a) Serotype I FECV strain RM (FJ938051).
b) Serotype II FIPV strain 79-1146 (DQ010921)
c) These primers were used for ascites of cat No. 58.
d) Specific names were not given for the primer in the original paper.
e) These primers were designed in this study.

Supplementaly Table 2. FCoV-positive samples and obtained 3c genes.

Nos. of cats	Age[a)]	Sample	Isolate	Serotype	Gene Length (base)	ORF length (base)[b)]	Accession No.	3c gene truncation	S (1058/1060)
1	4m	Ascites	FCoV/II/JP13/As/1/2014	II	712	672	LC316063	Truncated	II[d)]
5	1y 2m	Ascites	FCoV/I/JP40/As/5/2014	I	713	90	LC316064	Truncated	No mutation
6	9m	Pleural effusion	FCoV/I/JP13/Pe/6/2014	I	711	315	LC316065	Truncated	M1058L
7-1	3m	Pleural effusion	FCoV/I/JP40/Pe/7-1/2014	I	712	486	LC316066	Truncated	S1060A
7-2	3m	Ascites	FCoV/I/JP40/As/7-2/2014	I	714	336	LC316067	Truncated	M1058L
11	1y[c)]	Pleural effusion	FCoV/I/JP40/Pe/11/2014	I	714	0	LC316068	Truncated	S1060A
15	10m	Ascites	FCoV/I/JP40/As/15/2014	I	702	363	LC316069	Truncated	M1058L
17	7m	Ascites	FCoV/I/JP1/As/17/2014	I	711	252	LC316070	Truncated	M1058L
19	1y 7m	Pleural effusion	FCoV/I/JP40/Pe/19/2014	I	692	0	LC316071	Truncated	M1058L
22	2y 4m	Feces	FCoV/I/JP13/Fe/22/2014	I	714	714	LC316072	Intact	No mutation
23	5 m	Ascites	FCoV/I/JP40/As/23/2014	I	714	714	LC316073	Intact	S1060A
24	1y 2m	Feces	FCoV/I/JP14/Fe/24/2015	I	714	714	LC316074	Intact	No mutation
		Pleural effusion	FCoV/I/JP14/Pe/24/2015	I	702	138	LC316075	Truncated	M1058L
30	6m	Ascites	FCoV/I/JP15/As/30/2015	I	714	714	LC316076	Intact	M1058L
32	17y	Feces	FCoV/I/JP13/Fe/32/2015	I	714	714	LC316077	Intact	No mutation
		Ascites	FCoV/I/JP13/As/32/2015	I	711	282	LC316078	Truncated	M1058L
33	1y 2m	Ascites	FCoV/I/JP13/As/33/2015	I	705	0/234	LC316079	Truncated	M1058L
35	4m	Feces	FCoV/I/JP15/Fe/35/2015	I	714	714	LC316080	Intact	No mutation
		Ascites	FCoV/I/JP15/As/35/2015	I	687	519	LC316081	Truncated	M1058L
36	2y 6m	Ascites	FCoV/I/JP40/As/36/2015	I	685	111	LC316082	Truncated	S1060A
37	4y 6m	Feces	FCoV/I/JP14/Fe/37/2015	I	720	720	LC316083	Intact	No mutation
38	Unknown	Ascites	FCoV/I/JP40/As/38/2015	I	712	636	LC316084	Truncated	M1058L
39	3y 11m	Ascites	FCoV/I/JP40/As/39/2015	I	714	51	LC316085	Truncated	No mutation
40	5m	Feces	FCoV/I/JP15/Fe/40/2015	I	714	714	LC316086	Intact	No mutation
		Pleural effusion	FCoV/I/JP15/Pe/40/2015	I	703	102	LC316087	Truncated	M1058L
45	9y	Ascites	FCoV/I/JP40/As/45/2015	I	714	618	LC316088	Truncated	M1058L
48	6y	Ascites	FCoV/I/JP40/As/48/2015	I	714	0	LC316089	Truncated	M1058L
53	1y 5m	Ascites	FCoV/I/JP40/As/53/2015	I	678	402	LC316090	Truncated	M1058L
54	5m	Ascites	FCoV/I/JP13/As/54/2015	I	713	30	LC316091	Truncated	M1058L
55	1y	Blood	FCoV/I/JP40/Bl/55/2015	I	714	714	LC316092	Intact	M1058L
58	8m	Ascites	FCoV/II/JP38/As/58/2015	II	735	12	LC316093	Truncated	II[d)]
62	1y 2m	Pleural effusion	FCoV/I/JP14/Pe/62/2015	I	722	183	LC316094	Truncated	M1058L
70	2m	Feces	FCoV/I/JP15/Fe/70/2016	I	714	714	LC316095	Intact	No mutation
		Ascites	FCoV/I/JP15/As/70/2016	I	714	714	LC316096	Intact	M1058L
75	1y 2m	Ascites	FCoV/I/JP38/As/75/2016	I	708	708	LC316097	Truncated	M1058L
80	4m	Rectal swab	FCoV/I/JP38/Rs/80/2016	I	714	714	LC316098	Intact	No mutation
		Kidney	FCoV/I/JP38/Ki/80Ki-712/2016	I	712	486	LC316099	Truncated	M1058L
		Kidney	FCoV/I/JP38/Ki/80Ki-684/2016	I	684	486	LC316100	Truncated	
		Lymph node	FCoV/I/JP38/Ln/80Ln-712/2016	I	712	486	LC316101	Truncated	M1058L
		Lymph node	FCoV/I/JP38/Ln/80Ln-684/2016	I	684	486	LC316102	Truncated	
81	4m	Rectal swab	FCoV/I/JP38/Rs/81/2016	I	714	714	LC316103	Intact	No mutation
		Spleen	FCoV/I/JP38/Sp/81/2016	I	714	615	LC316104	Truncated	M1058L
		Lymph node	FCoV/I/JP38/Ln/81/2016	I	714	615	LC316105	Truncated	M1058L
82	4m	Rectal swab	FCoV/I/JP38/Rs/82/2016	I	714	630	LC316106	Truncated	M1058L
		Vitreous humor	FCoV/I/JP38/Vh/82/2016	I	714	630	LC316107	Truncated	M1058L
83	6y	Ascites	FCoV/I/JP14/As/83/2016	I	714	714	LC316108	Intact	M1058L
85	4m[c)]	Rectal swab	FCoV/I/JP38/Rs/85/2016	I	683	486	LC316109	Truncated	M1058L
		Ascites	FCoV/I/JP38/As/85/2016	I	712	486	LC316110	Truncated	M1058L
86	7m	Pleural effusion	FCoV/I/JP38/Pe/86/2016	I	704	357	LC316111	Truncated	M1058L
87	6m[c)]	Rectal swab	FCoV/I/JP38/Rs/87/2016	I	714	630	LC316112	Truncated	M1058L
		Kidney	FCoV/I/JP38/Ki/87/2016	I	714	630	LC316113	Truncated	M1058L
		Ascites	FCoV/I/JP38/As/87/2016	I	714	630	LC316114	Truncated	M1058L
88	6m	Feces	FCoV/I/JP38/Fe/88/2016	I	712	402	LC316115	Truncated	M1058L
90	13y	Ascites	FCoV/I/JP40/As/90/2016	I	720	324	LC316116	Truncated	M1058L
92	3y 6m	Rectal swab	FCoV/I/JP38/Rs/92/2017	I	714	714	LC316117	Intact	No mutation

a) Ages at which the clinical samples for this study were taken.

b) ORF lengths that were expected to be translated.

c) Estimated ages.

d) Serotype II

Supplementaly Table 3. FCoV-positive faecal samples of cats in a shelter.

Nos of cats	Isolate	Serotype	Gene length (base)[a)]	ORF length (base)[b)]	Accession No.	3c gene truncation	S (1058/1060)
S1	FCoV/I/JP14/Fe/S1/2014	I	714	714	LC316044	Intact	No mutation
S2	FCoV/I/JP14/Fe/S2/2015	I	714	714	LC316045	Intact	No mutation
S3	FCoV/I/JP14/Fe/S3/2015	I	714	714	LC316046	Intact	No mutation
S4	FCoV/I/JP14/Fe/S4/2015	I	714	714	LC316047	Intact	No mutation
S5	FCoV/I/JP14/Fe/S5/2015	I	714	714	LC316048	Intact	No mutation
S6	FCoV/I/JP14/Fe/S6/2015	I	714	714	LC316049	Intact	No mutation
S7	FCoV/I/JP14/Fe/S7/2016	I	714	714	LC316050	Intact	No mutation
S8	FCoV/I/JP14/Fe/S8/2016	I	714	714	LC316051	Intact	No mutation
S9	FCoV/I/JP14/Fe/S9/2017	I	714	714	LC316052	Intact	No mutation
S10	FCoV/I/JP14/Fe/S10/2017	I	711	711	LC316053	Truncated	No mutation
S11	FCoV/I/JP14/Fe/S11/2017	I	714	714	LC316054	Intact	No mutation
S12	FCoV/I/JP14/Fe/S12/2017	I	714	714	LC316055	Intact	No mutation
S13	FCoV/I/JP14/Fe/S13/2017	I	714	714	LC316056	Intact	No mutation
S14	FCoV/I/JP14/Fe/S14/2017	I	714	714	LC316057	Intact	No mutation
S15	FCoV/I/JP14/Fe/S15/2017	I	714	714	LC316058	Intact	No mutation
S16	FCoV/I/JP14/Fe/S16/2017	I	714	714	LC316059	Intact	No mutation
S17	FCoV/I/JP14/Fe/S17/2017	I	714	714	LC316060	Intact	No mutation
S18	FCoV/I/JP14/Fe/S18/2017	I	714	714	LC316061	Intact	No mutation
S19	FCoV/I/JP14/Fe/S19/2017	I	714	714	LC316062	Intact	No mutation

a) 3c gene length submitted to DDBJ.

b) ORF length that is expected to be translated.